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1. Your reference — RJE/RSG/PG4745

2. Patent application number
(The Patent Office will fill in his part) — 0201736.6 — 25 JAN 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Patents ADP number *(if you know it)* 4735870003

If the applicant is a corporate body, give the country/state of its incorporation

Glaxo Group Ltd
Glaxo Wellcome House, Berkeley Avenue,
Greenford, Middlesex UB6 0NN, Great Britain

United Kingdom

4. Title of the invention — DNA DOSAGE FORMS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Patents ADP number *(if you know it)* 8088437001

Corporate Intellectual Property

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Claim(s)	1
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11. We request the grant of a patent on the basis of this application

Signature _____ Date 25-Jan-02

R J Easeman

12. Name and daytime telephone number of person to contact in the United Kingdom: R J Easeman 020 80474407

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DNA dosage forms

The present invention relates to efficient devices for administration of DNA based pharmaceutical agents into the skin of the human body. In particular the present invention provides devices for administration of DNA vaccines into the skin. The present invention provides a DNA pharmaceutical agent delivery device having at least one skin-piercing element which comprises a support member coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent, and a stabilising agent that inhibits the degradative effects of free radicals. Preferably the solid pharmaceutical reservoir medium is an amorphous polyol, and preferably a carbohydrate such as trehalose or sucrose. Preferably the stabilising agent is a free radical scavenger or a metal ion chelator. Most preferably the solid vaccines of the present invention comprise a DNA plasmid, a free radical scavenger, and a metal ion chelator, in solid solution within a glassy sugar reservoir medium which is coated onto a support member. The devices of the present invention are storage stable, and only substantially release the DNA pharmaceutical after penetration of the skin piercing portion into the skin. In a preferred embodiment there is provided a microneedle device coated externally with the solid reservoir medium that releases the DNA pharmaceutical agent directly into the skin after piercing the stratum corneum. The DNA pharmaceutical delivery devices are proportioned such that agent is delivered into defined layers of the skin, and preferred delivery devices comprise skin-piercing portions that deliver the pharmaceutical agent into the epithelium or the dermis. Preferred reservoir media comprise sugars, and in particular stabilising sugars that form a glass such as lactose, raffinose, trehalose or sucrose. Furthermore, vaccine delivery devices for administration of vaccines into the skin are provided, methods of their manufacture, and their use in medicine.

The skin represents a significant barrier to external agents. A summary of human skin is provided in Dorland's Illustrated Medical Dictionary, 28th Edition. Starting from the external layers, working inwards, the skin comprises the epithelium comprising the stratum corneum, the viable epithelium, and underlying the epithelium is the dermis. The epithelium consists of five layers: Stratum corneum, Stratum lucidium, Stratum granulosum, Stratum spinosum, and Stratum basale. The *epithelium* (including all five layers) is the outermost non-vascular layer of the skin, and varies

between 0.07 and 0.12 mm thick (70-120 μm). The epithelium is populated with keratinocytes, a cell that produces keratin and constitutes 95% of the dedicated epidermal cells. The other 5% of cells are melanocytes. The underlying dermis is normally found within a range of 0.3 to about 3 mm beneath the surface of the stratum corneum, and contains sweat glands, hair follicles, nerve endings and blood vessels.

The stratum corneum dominates the skin permeability barrier and consists of a few dozen horny, keratinised epithelium layers. The narrow interstices between the dead or dying keratinocytes in this region are filled with crystalline lipid multilamellae. These efficiently seal the interstices between the skin or body interior and the surroundings by providing a hydrophobic barrier to entry by hydrophilic molecules. The stratum corneum being in the range of 30-70 μm thick.

Langerhans cells are found throughout the basal granular layer of the epithelium (stratum spinosum and stratum granulosum, (Small Animal Dermatology - Third Edition, Muller - Kirk – Scott, Ed: Saunders (1983)) and are considered to play an important role in the immune system's initial defence against invading organisms. This layer of the skin therefore represents a suitable target zone for certain types of vaccine.

Conventional modes for administration of pharmaceutical agents into or across the skin, most commonly by hypodermic needle and syringe, are associated with numerous disadvantages. Such disadvantages include pain, the requirement for trained professionals to administer the agent, and also the risk of needle-stick injuries to the administrator with the accompanying risk of infection with a blood born disease. As such, there is a need to improve the method of administration of all types of pharmaceutical into or through the skin.

A number of alternative approaches have been described in order to overcome the problems of administering agent across the stratum corneum, including various designs of skin patches. Examples of skin patches which deliver agent through the skin without physically penetrating the stratum corneum layer include that described in WO 98/20734 and WO 99/43350. Other approaches where the skin is not physically punctured include electrotransport, or iontophoretic devices where the passage of agent is enhanced by the application of an electrical current into the skin. Many such devices are described in the literature (examples of which include US 6,083,190; US 6.057,374; US 5,995,869; US 5,622,530). Potential disadvantages of

these types of non-penetration patches include the induction of significant sensitisation and discomfort during administration of the agent, and very poor uptake of antigen across the intact stratum corneum.

Other patches involving physical disruption or penetration of the skin have been described. Devices comprising liquid or solid reservoirs containing agent and a metal microblade patch have been described wherein the microblades physically cut through the stratum corneum to create pathways through which the agent can enter the epithelium. Such devices are described in WO 97/48440, WO 97/48442, WO 98/28037, WO 99/29298, WO 99/29364, WO 99/29365, WO 00/05339, WO 00/05166, and WO 00/16833. Other devices involving puncturing of the skin include US 5,279,544, US 5,250,023 and US 3,964,482.

Solid dosage forms comprising a pharmaceutical agents and a stabilising polyol, such as a sugar wherein the dosage forms are in the form of powders and trocars are described in WO 96/03978.

Supercoiled DNA in pharmaceutical preparations are known to degrade over time resulting in the loss of the supercoiled structure and associated formation of open circle or linear DNA structures (Evans *et al.*, 2000, Journal of Pharmaceutical Sciences, 89(1), 76-87; WO 97/40839). One mechanism by which this chain scission reaction may occur is oxidation of the DNA by free hydroxyl radicals produced from dissolved oxygen in the DNA solutions, a process that is catalysed by metal ions. The free radical formation reaction may be catalysed by several transition metal ions, the most common of which, however, are iron and copper ions (Fe^{+3}, Fe^{+2}, Cu^{+2} or Cu^{+1}; Evans *et al. supra*).

It has been shown that removal of trace metal ions from supercoiled DNA containing solutions with metal ion chelators, and/or mopping up free radicals in solution by non-reducing free radical scavengers stabilises the DNA in the supercoiled form and protects the DNA from oxidation (WO 97/40839).

Plasmid based delivery of genes, particularly for immunisation or gene therapy purposes is known. For example, administration of naked DNA by injection into mouse muscle is outlined in WO90/11092. Johnston et al WO 91/07487 describe methods of transferring a gene to veterbrate cells, by the use of microprojectiles that have been coated with a polynucleotide encoding a gene of interest, and accelerating the microparticles such that the microparticles can penetrate the target cell.

DNA vaccines usually consist of a bacterial plasmid vector into which is inserted a strong viral promoter, the gene of interest which encodes for an antigenic peptide and a polyadenylation/transcriptional termination sequences. The gene of interest may encode a full protein or simply an antigenic peptide sequence relating to the pathogen, tumour or other agent which is intended to be protected against. The plasmid can be grown in bacteria, such as for example *E.coli* and then isolated and prepared in an appropriate medium, depending upon the intended route of administration, before being administered to the host. Following administration the plasmid is taken up by cells of the host where the encoded protein or peptide is produced. The plasmid vector will preferably be made without an origin of replication which is functional in eukaryotic cells, in order to prevent plasmid replication in the mammalian host and integration within chromosomal DNA of the animal concerned. Information in relation to DNA vaccination is provided in Donnelly *et al* "DNA vaccines" *Ann. Rev Immunol.* 1997 15: 617-648, the disclosure of which is included herein in its entirety by way of reference.

The present invention overcomes the problems of the prior art and provides a device which is capable of administering and releasing the DNA agents efficiently into the skin, and also in which the DNA is stabilised such that it is released in its supercoiled form.

DESCRIPTION OF THE FIGURES.

FIG 1. shows the plasmids used in this study

A. pEGFP-C1, B. pGL3CMV, C. pVAC1.ova

FIG 2. shows 0.8% agarose gel electrophoresis using an E-gel, for analysis of supercoiled plasmid DNA, pEGFP-C1, after coating onto and immediate elution from sewing needles.

FIG 3. shows 1.2% agarose gel electrophoresis using an E-gel, for analysis of supercoiled plasmid DNA, pEGFP-C1, after coating onto and elution from sewing needles.

FIG. 4 shows 1.2% agarose gel electrophoresis using an E-gel, for analysis of supercoiled plasmid DNA, pGL3CMV, after coating onto and elution from sewing needles stored for varying time periods at 4^0C.

FIG. 5 shows 1% agarose gel electrophoresis, in the absence of ethidium bromide, (EtBr), for analysis of supercoiled plasmid DNA, pGL3CMV, after coating onto sewing needles.

FIG. 6 shows 1.2% agarose gel electrophoresis using an E-gel, for analysis of supercoiled plasmid DNA, pGL3CMV, after coating onto and immediate elution from sewing needles or 30G hypodermic needles.

FIG. 7 shows 1% agarose gel electrophoresis, in the absence of ethidium bromide, (EtBr), for analysis of supercoiled plasmid DNA, pVac1ova, (resuspended in a variety of different formulations at 5ug/ul), after coating onto sewing or hypodermic needles of different gauges and 'release' into agarose.

FIG. 8 shows 1% agarose gel electrophoresis, in the absence of ethidium bromide, (EtBr), for analysis of supercoiled plasmid DNA, pVac1ova,

FIG. 9 shows comparative luciferase activity, (48 hours post transfection), derived from plasmid pGL3CMV, delivered either intradermally, (ID, Fig. 9A), or intramuscularly, (IM, Fig. 9B), to mice, from DNA coated 30G hypodermic needles compared to controls of standard ID and standard IM delivery in saline.

The present invention provides a DNA agent delivery device having at least one skin-piercing element which comprises a support member coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent, and a stabilising agent that inhibits the degradative effects of free radicals. Alternatively, the skin piercing element may consist of the solid DNA pharmaceutical agent reservoir medium without the support member.

The solid pharmaceutical reservoir medium is an amorphous polyol, and preferably a carbohydrate such as trehalose or sucrose. Preferably the stabilising agent is a free radical scavenger or a metal ion chelator. Most preferably the solid vaccines of the present invention comprise a DNA plasmid, a free radical scavenger, and a metal ion chelator, in solid solution within an amorphous glassy reservoir medium which is coated onto a support member.

The skin piercing members that are loaded with the solid reservoir medium containing the agent to be delivered, after coating with the reservoir medium onto the support member, are long enough and sharp enough to pierce the stratum corneum of the skin. Once the pharmaceutical agent delivery device has been administered to the

surface of the skin, and the coated skin-piercing member or microneedle has pierced through the stratum corneum, the reservoir medium biodegrades thereby releasing the agent into the skin underlying the stratum corneum.

DNA vaccine delivery devices form a preferred aspect of the present invention. In such applications the agent to be delivered is a polynucleotide that encodes an antigen or antigens derivable from a pathogen such as micro-organisms or viruses, or may be a self antigen in the case of a cancer vaccine or other self antigen. The polynucleotide may be delivered alone or it may also comprise an agent to enhance uptake of the DNA into the cell, an adjuvant or other immunostimulant to improve and/or direct the immune response, and may also further comprise pharmaceutically acceptable excipient(s).

The DNA vaccine coated devices may be used for prophylactic or therapeutic vaccination and for priming and/or boosting the immune response.

Certain embodiments of the device described herein also have the significant advantage of being stored at room temperature thus reducing logistic costs and releasing valuable refrigerator space for other products.

The skin piercing protrusions which may be coated with reservoir medium to form preferred delivery devices of the present invention may be made of almost any material which can be used to create a protrusion that is strong enough to pierce the stratum corneum and which is safe for the purpose, for example the protrusions may be made of a metal, such as pharmaceutical grade stainless steel, gold or titanium or other such metal used in prostheses, alloys of these or other metals; ceramics, semi-conductors, silicon, polymers, plastics, glasses or composites.

The delivery devices may be in the form of a single needle or cannula, or may comprise multiple skin piercing elements in the form of a patch.

The patch generally comprise a backing plate from which depend a plurality of piercing protrusions such as microneedles or microblades. The piercing protrusions themselves may take many forms, and may be solid or hollow, and as such may be in the form of a solid needle or blade (such as the microblade aspects and designs described in McAllister *et al.*, *Annu. Rev. Biomed. Eng.*, 2000, 2, 289-313; Henry *et al.*, Journal of Pharmaceutical Sciences, 1998, 87, 8, 922-925; Kaushik *et al.*, *Anesth. Analg.*, 2001, 92, 502-504; McAllister *et al.*, *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.*, 26, (1999), Controlled Release Society, Inc., 192-193; WO 99/64580;

WO 97/48440; WO 97/48442; WO 98/28037; WO 99/29364; WO 99/29365; US 5,879,326, the designs of all of these documents, and the methods of manufacture of the microblade arrays being incorporated herein by reference). Alternatively the piercing protrusions may be in the form of a microneedle having a hollow central bore. In this last embodiment, the central bore may extend through the needle to form a channel communicating with both sides of the microneedle member (EP 0 796 128 B1). Solid microneedles and microblades are preferred.

The length of the skin-piercing member for administration of the DNA into the skin may be varied depending on which anatomical location the patch is to be administered and which layer of skin it is desired to administer the pharmaceutical agent in the vaccinee species. Typically between 1μm to 3mm, preferably between 1 μm and 1mm, preferably between 50μm and 600μm, and more preferably between 100 and 400μm. The length of the skin-piercing member may be selected according to the site chosen for targeting delivery of the agent, namely, preferably, the dermis and most preferably the epidermis. The skin-piercing members of the devices of the present invention may be take the form of, and be manufactured by the methods described in US 5,879,326, WO 97/48440, WO 97/48442, WO 98/28037, WO 99/29298, WO 99/29364, WO 99/29365, WO 99/64580, WO 00/05339, WO 00/05166, or WO 00/16833; or McAllister *et al.*, *Annu. Rev. Biomed. Eng.*, 2000, 2, 289-313; Henry *et al.*, Journal of Pharmaceutical Sciences, 1998, 87, 8, 922-925; Kaushik *et al.*, *Anesth. Analg.*, 2001, 92, 502-504; McAllister *et al.*, *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.*, 26, (1999), Controlled Release Society, Inc., 192-193.

The most preferred microblade devices to be coated with the pharmaceutical agent reservoir medium to form devices of the present invention are described in WO 99 48440 and Henry *et al.*, Journal of Pharmaceutical Sciences, 1998, 87, 8, 922-925, the contents of both are fully incorporated herein.

The devices of the present invention preferably comprise a plurality of skin-piercing members, preferably up to 1000 members per device, more preferably up to 500 skin-piercing members per device.

Where the piercing protrusion is solid, it may flat or may have a circular or polygonal cross section. The protrusions can have straight or tapered shafts and may be flat or circular, or other polygonal shape, in cross section. For example, the microblades may

have a curved blade or be formed into a V-section groove. Alternatively the protrusions may have more complex shapes to enhance adherence and fluid dynamics such as a five pointed star.

The skin-piercing members may be integral with the backing plate or may be attached thereto. In the case where the protrusions may be attached to the plate, the piercing protrusion may be formed of the reservoir medium. Such devices may be made by formed by drawing or extruding a molten reservoir medium containing the agent into fine points. For instance, molten reservoir medium could be cast directly onto a backing plate through a multipore head, where the hot extrudate cools and sticks to the plate. When you draw back the extrudate a series of pointed ends is formed.

As a general feature of any piercing protrusion shape, in order to improve reservoir adherence after coating, the surface of the protrusion may be textured. For example, the surface may be coarse grained, rippled or ribbed. In addition, solid microblades may further comprise through holes, such that the reservoir may dry therein and create a reservoir tie, to hold the reservoir onto the blade more securely. In certain embodiments, including highly soluble and friable lyophilised formulations, it is preferred that the friable reservoir may be entirely held within such holes thereby protected from breakage during puncture of the skin.

In an alternative embodiment the piercing protrusions may be separable from the base member. For example, in the embodiment where the piercing protrusions (or at least the tips thereof) is the reservoir itself, after penetration of the skin the piercing protrusions separates from the base support thus allowing the patch to be removed from the skin, whilst leaving the reservoir behind in the skin. The separation of the reservoir from the backing plate may be by physical shearing or by biodegradation of part of the needles adjacent the backing plate.

One embodiment of this may be to cast the microprotrusion tips out of a relatively poorly soluble disaccharide reservoir medium (containing a dispersion of the agent to be delivered) followed by casting the remaining portion of the microprotrusion and backing plate out of a relatively easily soluble material. Once inserted into the skin, the relatively easily soluble microprotrusion shaft would degrade away, thereby allowing the patch to be removed from the skin, whilst leaving

the tips within the skin. The tips, remaining in the skin can then slowly release the agent by slower biodegradation.

The polyol biodegradable agent reservoir may be any made from any medium that fulfils the function required for the present invention. The reservoir must be capable of adhering to the microprotrusion to a sufficient extent that the reservoir remains physically stable and attached during prolonged storage, and also remains substantially intact during the administration procedure when the coated microprotrusion pierce the stratum corneum. The reservoir must also be capable of holding or containing a suspension or solution of agent to be delivered in any dry or partially dry form, which is released into the skin during biodegradation of the reservoir medium.

Biodegradation of the medium in the sense of the present invention means that the reservoir medium changes state, such that changes from its non-releasing to its releasing states whereby the agent enters into the skin. The release of the active agent may involve one or more physical and/or chemical processes such as hydration, diffusion, phase transition, crystallisation, dissolution, enzymatic reaction and/or chemical reaction. Depending on the choice of reservoir medium, biodegradation can be induced by one or more of the following: water, body fluids, humidity, body temperature, enzymes, catalysts and/or reactants. The change of the reservoir medium may therefore be induced by hydration, and warming associated with the higher humidity and temperature of the skin. The reservoir medium may then degrade by dissolution and/or swelling and/or change phase (crystalline or amorphous), thereby disintegrating or merely increase the permeation of the medium.

Preferably the medium dissolves, and is metabolised or expelled or excreted from the body, but the reservoir may alternatively remain attached to the skin-piercing member to be removed from the skin when the device is removed. Release of the agent by dissolution of the reservoir medium is preferred.

Suitable polyol reservoir media are as sugars, polysaccharides, substituted polyols such as hydrophobically derivatised carbohydrates, amino acids, biodegradable polymers or co-polymers such as poly(hydroxy acid)s, polyahhydrides, poly(ortho)esters, polyurethanes, poly(butyric acid)s, poly(valeric acid)s, and poly(lactide-co-caprolactone)s, or polylactide co-glycolide. The coating of the

microblades may be in the amorphous or crystalline state and may also be partially amorphous and partially crystalline.

Particularly preferred reservoir media are those that stabilise the agent to be delivered over the period of storage. For example, antigen or agent dissolved or dispersed in a polyol glass or simply dried in a polyol are storage stable over prolonged periods of time (US 5,098,893, US 6,071,428; WO 98/16205; WO 96/05809; WO 96/03978; US 4,891,319; US 5,621,094; WO 96/33744). Such polyols form the preferred set of reservoir media.

Preferred polyols include sugars, including mono, di, tri, or oligo saccharides and their corresponding sugar alcohols. Suitable sugars for use in the present invention are well known in the art and include, trehalose, sucrose, lactose, fructose, galactose, mannose, maltulose, iso-maltulose and lactulose, maltose, or dextrose and sugar alcohols of the aforementioned such as mannitol, lactitol and maltitol. Sucrose, Lactose, Raffinose and Trehalose are preferred.

Preferably the DNA and stabilising agent are in a solid solution within the amorphous, and preferably glassy reservoir medium.

It is preferred that the reservoir medium forms an amorphous glass upon drying. The glass reservoir may have any glass transition temperature, but preferably it has a glass transition temperature that both stabilises the pharmaceutical agent during storage and also facilitates rapid release of the agent after insertion of the reservoir into the skin. Accordingly, the glass transition temperature is greater than 30-40°C, but most preferably is around body temperature (such as, but not limited to 37-50°C).

Preferably the solid pharmaceutical reservoir medium contains a DNA condensing agent for example spermidine or PEI (polyethyleneimine).

In another related embodiment of the present invention the devices may be electroporation devices. For example US 6261281 describes liquid intramuscular DNA vaccination followed by insertion of electrodes to pass an electric current across the muscle cells to enhance uptake of the DNA into the cells. WO 00/44438 describes needle patches coated with DNA in the absence of a reservoir medium, the metal needles being used as electrodes. Accordingly there is provided a electroporation device comprising a plurality of skin piercing elements which comprise a support member coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent, and a stabilising agent that inhibits the degradative effects of

free radicals. One preferred embodiment of this are the devices described in WO 00/44438 (the contents of which are incorporated herein by reference) the needles of which are coated with an amorphous reservoir medium containing a DNA vaccine and a stabilising agent that inhibits the degradative effects of free radicals.

The preferred reservoir media used to coat the skin-piercing members of the devices are those that release the pharmaceutical agent over a short period of time. The preferred reservoir formulations release substantially all of the agent within 10 minutes, more preferably within 5 minutes, more preferably within 2 minutes, more preferably within 1 minute, and most preferably within 30 seconds of insertion into a 1% agarose gel. Such fast releasing reservoirs can be achieved, for example, by thin coatings of amorphous glass reservoirs, particularly fast dissolving/swelling glassy reservoirs having low glass transition temperatures. It will be clear to the man skilled in the art that a low glass transition temperature can be achieved by selecting the appropriate glass forming sugar, and/or increasing humidity and/or ionic strength of the glass. Additionally, increased speed of dissolution of glass reservoirs may also be achieved by warming the device before or during application to the skin, or by adding additional agents to decrease the dissolution time.

The formulations of the present invention are substantially glassy formulations, although mixed formulations comprising amorphous and crystalline states are also part of the present invention.

One major advantage of the present invention is the fact that the DNA is stabilised so that upon release, it is largely in its supercoiled form. Amongst other factors this stability is primarily a result of the encapsulation of the DNA in the amorphous or glassy reservoir medium, and also the presence of agents to counter the effects of free radicals. This second mechanism is particularly important in embodiments where the reservoir is coated onto a supply of iron ions, such as a stainless steel needle or microneedle array.

Plasmid DNA stability can be defined in a number of ways and can be a relative phenomenon determined by the conditions of storage such as pH, humidity and temperature. For storage in the presence of iron ions on the coated reservoir, preferably >50% of plasmid remains supercoiled, (ccc, covalently closed circular), upon storage for 3 months at 4^0C. More preferably, under the storage conditions

described, >60% of plasmid remains ccc and more preferably, under these storage conditions, >90% of plasmid remains ccc for 3 months at 4^0C. For coating on to non – metal ion based needles or microneedles, the stability of plasmid DNA would be preferably >60% and more preferably 80% and most preferably >90% ccc after 3 months storage at 4^0C. More preferably, under these storage conditions, >90% of plasmid remains ccc for 1 year at 4^0C, and more preferably >90% of plasmid remains ccc for 2 years at 4^0C. Most preferably the above DNA stability is achieved over the same time periods at 25^0C.

Studies to determine plasmid stability are well known to those skilled in the art and are described in (Evans *et al., Supra;* WO 97/40839). These include techniques to measure and quantify the percentage of supercoiled, ccc, plasmid DNA either by agarose gel electrophoresis, anion exchange HPLC, (Ferreira, G. *et al.,* 1999, *Pharm. Pharmacol. Commun.*, 5, pp57-59), or capillary gel electrophoresis, (Schmidt *et al.*, 1999, *Anal. Biochem.*, 274, 235-240).

The reservoir mediums of the present invention contain a stabilising agent that inhibits the degradative effects of free radicals. Preferred stabilising agents include stabilising metal ion chelating agents, while preferred metal ion chelating agents include inositol hexaphosphate, tripolyphosphate, succinic and malic acid, ethylenediamine tetraacetic acid (EDTA), tris (hydroxymethyl) amino methane (TRIS), Desferal, diethylenetriaminepentaacetic acid (DTPA) and ethylenediamindihydroxyphenylacetic acid (EDDHA). Other preferred stabilising agents are non-reducing free radical scavengers, and preferably such as agents are ethanol, methionine or glutathione. Other suitable chelators and scavengers (and those which are not suitable) may be readily identified by the man skilled in the art by routine experimentation (as described in WO 97/40839).

The amounts of the components present may be determined by the man skilled in the art, but generally are in the range of 0.1-10mM for the metal ion chelators, Ethanol is present in an amount up to about 5% (v/v), methionine is present at about 0.1 to 100mM and Glutathione is present at about 0.1 to 10% (v/v).

Particularly preferred formulations which may be combined with the DNA and the polyols: sucrose or trehalose in demetalated water or Phosphate or Tris based buffers and then dried onto the devices of the present invention are:

A. 10mM methionine and 2.9% ethanol

B. 3.7% ethanol and 1mM EDTA

C. 100mM Tris, 1mM EDTA and 10mM methionine and 2.9% ethanol

D. 100mM Tris, 1mM EDTA and 10mM methionine

E. 100mM Tris, 1mM EDTA and 2.9% ethanol

The preferred solid reservoir media in the devices of the present invention contain a metal ion chelating agent or a non-reducing free radical scavenger. Most preferably the solid reservoir media in the devices of the present invention contain both a metal ion chelating agent and a non-reducing free radical scavenger.

In addition to these stabilising agents, further steps may be taken to enhance the stability of the DNA in the solid vaccines. For example, the formulations may be made using solutions which themselves were demetalated before use (for example by using commercially available demetalating resin such as Chelex 100 from Biorad) and/or the formulation may be finalised in a high pH (such as pH 8-10)

Other suitable excipients which may be included in the formulation include buffers, amino acids, phase change inhibitors ('crystal poisoners') which may be added to prevent phase change of the coating during processing or storage or inhibitors to prevent deleterious chemical reactions during processing or storage such Maillard reaction inhibitors like amino acids.

The formulations comprising the agent to be delivered and biodegradable reservoir medium are preferably mixed in aqueous solution and then dried onto the microprotrusion member or the formulation could be melted and then applied to the microprotrusion member. A preferred process for coating the skin-piercing members comprises making an aqueous solution of vaccine antigen and water soluble polyol (such as trehalose), followed by coating the solution onto the microblades by dipping the member into the solution one or more times followed by drying at ambient temperature or lyophilisation to give a porous coating. In this process it is preferred that the initial solution of water soluble polyol or sugar is viscous, such as the viscosity achieved from 40% sugar.

Alternatively, minute picolitre volumes of solution or melted formulation may be sprayed onto individual blades by technology commonly used in the art of bubble-jet printers, followed by drying. An alternative method would be to prepare microspheres or microparticles or powders of amorphous formulation containing

polyol such as sugar, using techniques known in the art (such as spray drying or spray freeze drying or drying and grinding) and by controlling the moisture content to achieve a relatively low glass transition temperature (for example 30°C), followed by spraying or dipping to bring the microphores or microparticles or powders into contact with a microprotrusion member heated to a temperature above that of the glass transition temperature of the microsphere (for example 45°C). The coated particles would then melt and adhere to the microprotrusion member and then dry or the coated microblade member would be further dried (to remove residual moisture content) thereby increasing the glass transition temperature of the reservoir medium suitable for storage.

Alternatively, the microneedle member may be coated using a freeze coating technique. For example, the temperature of the microneedle member may be lowered below that of the freezing point of water (for example by dipping in liquid nitrogen) and then aqueous solutions of the reservoir medium and agent my be sprayed onto the cold microneedles, or the microblade may be dipped into the solution of agent. In this way the agent and reservoir medium rapidly adheres to the microneedle member, which can then be sublimed by lyophilisation, or evaporated at higher temperatures, to dry the reservoir coating.

Another method to coat the microneedle members is to dip the microneedles in a solvent, such as water (optionally comprising a surfactant to ensure good contact) then dipping wetted blades in a powdered form of the reservoir medium which is soluble in the solvent, followed by drying to remove the solvent.

In a preferred embodiment of the invention there is provided a process for coating a microblade with a viscous solution of reservoir forming medium which is sufficiently fluid to allow sterile filtration through a 220 nm pore membrane. Accordingly there is provided a vaccine formulation comprising antigen in a filterable viscous sugar solution formulation. Preferred examples of such filterable viscous sugar solutions are solutions of between about 20 to about 50 % sugar (weight/volume of the final vaccine formulation prior to drying). More preferably the viscous filterable sugar solutions are in the range of about 30% to about 45% sugar, and most preferable are about 40% (weight sugar/volume of the final vaccine formulation prior to drying). In this context the most preferred sugar solutions comprise sucrose, raffinose, trehalose or lactose.

Using these techniques each skin piercing member may be loaded with relatively high amounts of pharmaceutical agent. Each piercing member preferably being loaded with up to 500 ng of DNA pharmaceutical, more preferably up to 1 μg of pharmaceutical DNA, more preferably up to 5μg of pharmaceutical DNA and most preferably up to 10μg of pharmaceutical DNA.

Preferably the vaccine formulations of the present invention contain DNA that encode an antigen or antigenic composition capable of eliciting an immune response against a human pathogen, which antigen or antigenic composition is derived from HIV-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Rotavirus (including live-attenuated viruses), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or a derivative thereof), hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins or derivatives thereof), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18, ..), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus (whole live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or Vero cells or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof), or derived from bacterial pathogens such as *Neisseria spp,* including *N. gonorrhea* and *N. meningitidis* (for example capsular polysaccharides and conjugates thereof, transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *S. pyogenes* (for example M proteins or fragments thereof, C5A protease, lipoteichoic acids), *S. agalactiae, S. mutans; H. ducreyi; Moraxella spp, including M catarrhalis, also known as Branhamella* catarrhalis (for example high and low molecular weight adhesins and invasins*);* *Bordetella spp, including B. pertussis* (for example pertactin, pertussis toxin or derivatives thereof, filamenteous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis* (for example ESAT6, Antigen 85A, -B or -C), *M. bovis, M. leprae, M. avium, M.*

paratuberculosis, M. smegmatis; Legionella spp, including *L. pneumophila; Escherichia spp,* including *enterotoxic E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic *E. coli,* enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp,* including *V. cholera* (for example cholera toxin or derivatives thereof); *Shigella spp,* including *S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp,* including *Y.* enterocolitica (for example a Yop protein) , *Y. pestis, Y. pseudotuberculosis; Campylobacter spp,* including *C. jejuni* (for example toxins, adhesins and invasins) and *C. coli; Salmonella spp,* including *S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp.,* including *L. monocytogenes; Helicobacter spp,* including *H.* pylori (for example urease, catalase, vacuolating toxin); *Pseudomonas spp,* including *P. aeruginosa; Staphylococcus spp.,* including *S. aureus, S. epidermidis; Enterococcus spp.,* including *E. faecalis, E. faecium; Clostridium spp.,* including *C.* tetani (for example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof), *C. difficile* (for example clostridium toxins A or B and derivatives thereof); *Bacillus spp.,* including *B. anthracis* (for example botulinum toxin and derivatives thereof); *Corynebacterium spp.,* including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.,* including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB), *B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii; Ehrlichia spp.,* including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia spp,* including *R. rickettsii; Chlamydia spp., including C. trachomatis* (for example MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins), *C. psittaci; Leptospira spp.,* including *L. interrogans; Treponema spp.,* including *T. pallidum* (for example the rare outer membrane proteins), *T. denticola, T. hyodysenteriae;* or derived from parasites such as *Plasmodium spp.,* including *P. falciparum; Toxoplasma spp.,* including *T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp.,* including *E. histolytica; Babesia spp.,* including *B. microti; Trypanosoma spp.,* including *T. cruzi; Giardia spp.,* including *G. lamblia; Leshmania spp.,* including *L. major; Pneumocystis spp.,* including *P. carinii; Trichomonas spp.,* including *T. vaginalis; Schisostoma spp.,* including *S. mansoni,* or derived from yeast such as *Candida spp.,* including *C. albicans;*

Cryptococcus spp., including *C. neoformans*. Other preferred bacterial vaccines comprise antigens derived from *Haemophilus spp., including H. influenzae type B* (for example PRP and conjugates thereof), *non typeable H. influenzae,* for example OMP26, high molecular weight adhesins, P5, P6, protein D and lipoprotein D, and fimbrin and fimbrin derived peptides (US 5,843,464)

Vaccines of the present invention, may advantageously also include an adjuvant. Suitable adjuvants for vaccines of the present invention comprise those adjuvants that are capable of enhancing the antibody responses against the IgE peptide immunogen. Adjuvants are well known in the art (Vaccine Design – The Subunit and Adjuvant Approach, 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X). Preferred adjuvants for use with immunogens of the present invention include aluminium or calcium salts (hydroxide or phosphate).

In an embodiment of the invention, a polynucleotide is administered/delivered as "naked" DNA, for example as described in Ulmer et al., *Science 259*:1745-1749, 1993 and reviewed by Cohen, *Science 259*:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto inert metallic beads, such as gold, or biodegradable beads, which are efficiently transported into the cells; or by using other well known transfection facilitating agents, such as Calcium Phosphate.

DNA may also be administered in conjunction with a carrier such as, for example, liposomes, and everything being entrapped in the reservoir medium. Typically such liposomes are cationic, for example imidazolium derivatives (WO95/14380), guanidine derivatives (WO95/14381), phosphatidyl choline derivatives (WO95/35301), piperazine derivatives (WO95/14651) and biguanide derivatives.

Examples of suitable pharmaceutically acceptable excipients include water, phosphate buffered saline, isotonic buffer solutions.

It is an intention of the present invention to administer agent or vaccine into the skin rapidly and with high yield of administration. This may be even further enhanced by a number of means, comprising the use of highly soluble carbohydrates as the reservoir medium, and also by agitating and/or heating the microneedle member during administration.

The amount of expressible DNA in each vaccine dose is selected as an amount which induces an immunoprotective response without significant adverse side effects in typical vaccinees. Such amount will vary depending upon which specific DNA construct is employed, however, it is expected that each dose will generally comprise 1-1000 μg of DNA, preferably 1-500 μg, more preferably 1-100 μg, of which 1 to 50μg is the most preferable range. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

The formulations of the present invention may be used for both prophylactic and therapeutic purposes. Accordingly, the present invention provides for a method of treating a mammal susceptible to or suffering from an infectious disease or cancer, or allergy, or autoimmune disease. In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine. Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

The present invention is exemplified by, but not limited to, the following examples.

Example 1, *Demonstration of coating of needles with lyophilised plasmid DNA.*

1.1 Plasmid preparation and formulations.

The plasmids used in this study are all shown in Figure 1.

pEGFP-C1 is a GFP expression vector, (Clontech, Palo Alto, California, USA).

pGL3CMV is a luciferase expression vector based upon pGL3 Basic, (Promega Corporation., Madison, Wisconsin, USA), where the CMV immediate early promoter drives luciferase expression.

pVAC1.ova is a chicken ovalbumin expression plasmid, constructed by ligating PCR amplified cDNA encoding chicken ovalbumin from pUGOVA, into the expression vector pVAC1. pVAC1 is a modification of the mammalian expression vector, pCI, (Promega), where the multiple cloning site, from EcoRI to Bst ZI, has been replaced by the EMCV IRES sequence flanked 5' by unique Nhe I, Rsr II and Xho I and 3' by

unique Pac I, Asc I and Not I restriction enzyme sites, amplified from pGL3Basic, (Promega). Supercoiled plasmid DNA, (low endotoxin), was purified on a large scale, aproximately 100mg yield, to high purity using a combination of alkaline SDS lysis, ultrafiltration and anion exchange column chromatography.

Plasmids were resuspended in TE, (10mM TrisHCl, 1mM EDTA), pH 8.0 at 1ug / ul. And determined as >95% supercoiled upon analysis by agarose gel electrophoresis.

Plasmids were formulated in a variety of solutions, for coating needles, by a standard large-scale ethanol precipitation procedure. The precipitated DNA was resuspended directly into the aqueous formulation solutions at concentrations of 0.5 to 12 ug/ul, (See Chapter 1, Molecular Cloning: A Laboratory Manual, Sambrook, J. *et al.*, 2nd Edition, 1989, CSH laboratory Press, Cold Spring Harbor, New York, USA).

1.2 Needles and coating procedures.

Size 8 sewing needles, (A817, Miliners, Milward, Coats Crafts, UK), were obtained from the John Lewis partnership, PLC, (London, UK.). Hypodermic needles, Microlance 3, 30G, 26G, 25G and 21G were obtained from Becton and Dickinson, (New Jersey, USA).

Sewing needles were coated by dipping once for 30 to 60 seconds into 1.5 ml of plasmid formulation, needle suspended from the rubber stopper of a 2ml glass lyophilisation vial, such that 1.0 to 1.5cm of the sewing needle was coated. Hypodermic needles were similarly coated being manually held in place with a 1ml syringe to balance in 1.5ml plasmid formulations in 2ml plastic screw capped tubes, Sarstedt, (Numbrecht, Germany).

Acid treated needles were dipped for 5 seconds in concentrated hydrochloric acid, (HCl), followed by distilled water washing and air drying on paper towels. Needles were then stored in a sterile glass beaker and subject to a single wet autoclave cycle for 20 minutes at 15 psi. Needles were then dried in a fume hood and stored at room temperature.

1.3 Lyophilisation and elution of plasmid DNA.

Coated needles were lyophilised under vacuum for a minimum of one hour at -45^{0}C or below using a Modulyo 4K Freezer Dryer, (Edwards, Crawley, UK). DNA coated, lyophilised needles were either used immediately or stored sealed at 4^{0}C.

Plasmid DNA was eluted from coated needles by shaking for 30 minutes in 0.5ml of 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, 2.9% ethanol in 2ml plastic screw capped tubes, Sarstedt, at room temperature. Plasmid DNA was then recovered by standard ethanol precipitation, (Sambrook, J. *et al., supra*), and resuspended in 20 to 30 ul of 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, 2.9% ethanol for application on to agarose gels.

1.4 Agarose gel electrophoresis and agarose gel 'needle release assay'.

Plasmid DNA and that eluted from needles after coating was routinely run on either 0.8% or 1.2% agarose E-gels containing ethidium bromide according to manufactures instructions, Invitrogen Corporation, (Carlsbad, California, USA).

To monitor the level of release of plasmid DNA from coated needles directly into agarose gels, 2.5cm thick, 1% agarose, (Ultra Pure, Life Technologies, Paisley, Scotland, UK), gels were poured. Needles were inserted into the agarose just behind the well to allow for plasmid DNA release from the needle into the agarose. Gel electrophoresis was then performed without ethidium bromide or other DNA visualisation agents at 100V, 100mA for 1 to 2 hours, (see Chapter 6 in Sambrook, J. *et al., supra*). DNA was then visualised by staining with with SYBR Gold, according to manufacturer's instructions, (Molecular Probes, Eugene, Oregon, USA.). Gel analysis was performed using the Visionworks package on the UVP8000 gel analysis system, (UVP Life Sciences, Cambridge, UK.).

1.5 Coating of sewing needles with high concentrations of DNA in aqueous solution containing stabilising agents.

Initial experiments to investigate the feasibility of coating sharp surfaces with plasmid DNA from solution were performed on acid treated sewing needles. Size 8 sewing needles were dipped once for 2-3hrs in plasmid DNA solutions ranging from 0.5ug/ul to 5ug/ul prepared in either water or 0.85% saline. Needles were carefully removed and lyophilised and then stored at 4^0C, sealed for 60 hrs before DNA was eluted, by vigorous shaking in 500ul TE for 30 min. DNA was concentrated by ethanol preciptation and analysed by gel electrophoresis. DNA, <100ng, partially degraded, was recovered from the 5ug/ul saline sample. No other samples showed sufficient DNA to be detected on an agarose gel, (data not shown). It was thought

likely that the acid treatment procedure was generating a combination of ferrous and ferric metal ions that were adversely affecting short-term plasmid DNA stability, (Evans *et al. Supra;* WO 97/40839).

To improve the needle coating procedure and reduce DNA degradation plasmid was formulated in aqueous solution in the presence of a number of chemicals, (eg. chelators and free radical scavengers), shown to increase plasmid stability in the presence of metal ions, (Evans *et al. Supra;* WO 97/40839). Sewing needles were also cleaned with methanol and air-dried to reduce metal ion formation. Plasmid DNA solutions were prepared at very high concentration 5ug/ul or 10ug/ul in 100mM Tris, 1mM EDTA, 10mM methionine, 2.9% ethanol pH 8.0, (formulation 2). Size 8 sewing needles were coated with DNA by dipping in solution, overnight at 4^0C, once and needles were carefully removed and lyophilised and then DNA was immediately eluted, concentrated and analysed by gel electrophoresis.

Data from such an analysis is shown in Figure 2, where coating of acid treated and methanol washed needles is compared.

The groups tested were run in lanes:
1) 1ug of 1kb DNA ladder, (Promega). 2) Eluted pEGFP-C1 plasmid from a 5ug/ul stock coated on to HCl treated sewing needles. 3) Eluted pEGFP-C1 plasmid from a 10ug/ul stock coated on to HCl treated sewing needles. 4) Eluted pEGFP-C1 plasmid from a 5ug/ul stock coated on to methanol washed sewing needles. 5) Eluted pEGFP-C1 plasmid from a 10ug/ul stock coated on to methanol washed sewing needles. 6) pEGFP-C1 plasmid standard – 0.5ug. 7) pEGFP-C1 plasmid standard – 1.0ug, 8) pEGFP-C1 plasmid standard – 5.0ug, 9) pEGFP-C1 plasmid standard – 10.0ug

In the absence of excess metal ions generated by acid treatment, non-degraded DNA, 1-2ug, was recovered from the 5ug/ul sample whereas the 10ug/ul sample yielded $\geq$ 10ug. This established the principle that DNA could be coated and lyophilised on to sharp objects such as sewing needles and eluted largely intact.

Example 2, *Stability of high concentrations of plasmid after coating on to needles.*

The reproducibility of needle coating and short-term stability of lyophilised plasmid DNA stored at 4^0C on needles was then investigated. Plasmid DNA solutions of 5ug/ul or 10ug/ul in 100mM Tris, 1mM EDTA, 10mM methionine, 2.9% ethanol

pH 8.0, (formulation 2), were coated onto size 8 sewing needles, lyophilised and the DNA was either immediately eluted or stored at 4^0C for 1 - 4 days. Data for such an analysis is displayed in Figure 3, where duplicate needles are shown, stored for each time point:

(A) Eluted pEGFP-C1 plasmid from a 5ug/ul stock coated on to methanol washed sewing needles.
(B) Eluted pEGFP-C1 plasmid from a 10ug/ul stock coated on to methanol washed sewing needles.
In each gel the lanes represent: 1) pEGFP-C1 plasmid standard – 5.0ug **(A)**, 10.0ug **(B)**. 2) 1ug of 1kb DNA ladder, (Promega). 3) & 4) Immediate elution after coating. 5) & 6) Elution after storage on sewing needles at 4^0C for 16 hours. 7) & 8) Elution after storage on sewing needles at 4^0C for 40 hours. 9) & 10) Elution after storage on sewing needles at 4^0C for 64 hours. 11) & 12) Elution after storage on sewing needles at 4^0C for 88 hours.

Large amounts of plasmid, up to 5ug, can be retained largely intact on coated, lyophilised needles and eluted after 66 to 88 hours storage at 4^0C. However, coating, elution or storage was found to be somewhat variable, with the 10ug/ul sample showing the least variance. Conversion of covalently closed circular, (ccc), DNA, (bottom plasmid band, Fig. 3) to open circular, (oc), (top plasmid band, Fig. 3), seemed to be occurring over time upon storage. These issues were addressed with further improved formulations.

Example 3, *Improved short–term stability of high concentrations of plasmid after coating on to needles.*

Further formulations of plasmid, in 100mM Tris, 1mM EDTA, 10mM methionine, 2.9% ethanol pH 8.0, (formulation 2), but with the additional presence of a low sugar (17.5%) "LS" or high sugar (40%) "HS" percentages of sugars, either trehalose or sucrose, were evaluated for needle coating and short-term stability after lyophilisation. Agarose gel electrophoresis of eluted DNA from such an analysis is shown in Figure 4. Formulations containing 17.5 % sucrose, (LS) or trehalose, (LT),

(plasmid DNA at 5ug/ul), or 40% sucrose, (HS, plasmid DNA at 12ug/ul) or trehalose, (HT, plasmid DNA at 5ug/ul), were compared for needle coating, elution and DNA integrity.

Fig. 4 shows:

(A) Eluted pGL3CMV plasmid from formulation (HS) or formulation (HT), coated on to methanol washed sewing needles.

(B) Eluted pGL3CMV plasmid from formulations (LS) or (LT), coated on to methanol washed sewing needles.

With the lanes in each diagram illustrating: 1) 1ug of 1kb DNA ladder, (Promega). 2) Immediate elution after coating, HS: (A), LS: (B). 3) Elution after storage on sewing needles at 4^0C for 16 hours, HS: (A), LS: (B). 4) Elution after storage on sewing needles at 4^0C for 40 hours, HS: (A), LS: (B). 5) Elution after storage on sewing needles at 4^0C for 64 hours, HS: (A), LS: (B). 6) Immediate elution after coating, HT: (A), LT: (B). 7) Elution after storage on sewing needles at 4^0C for 16 hours, HT: (A), LT: (B). 8) Elution after storage on sewing needles at 4^0C for 40 hours, HT: (A), LT: (B). 9) Elution after storage on sewing needles at 4^0C for 64 hours, HT: (A), LT: (B).

The presence of low and high concentrations of sugars, the latter particularly, (see Fig 4a), improved reproducibility and stability of lyophilised DNA coated onto needles. There was no obvious increase in the proportion of open circular plasmid DNA upon storage for 3 days at 4^0C, in the presence of sugars and plasmid DNA remained as largely ccc format.

Example 4, *Release into agarose gels of high concentrations of plasmid from coated needles.*

Experiments were performed to look at the rate of release or 'delivery' of lyophilised plasmid DNA, from the high sugar formulations, (HS, example 3), coated onto sewing needles, upon re-hydration. Stock plasmid DNA was resuspended in 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, 2.9% ethanol, (formulation 2), additionally containing 40% sucrose, (HS), at 12ug/ul or trehalose, (HT), at 5ug/ul for needle coating. DNA 'release' was achieved by placing the needles, freshly coated with lyophilised plasmid DNA, into thick, 1% agarose gels, just above the gel wells, for increasing increments of time from 15 sec. to 15 min. and

performing electrophoresis. Gels were then stained with SYBR gold, the most sensitive DNA stain available, (see, Fig. 5A). Any remaining plasmid was eluted from the needles after 'stabbing' in to agarose and precipitated and analysed in SYBR gold stained agarose gels, as above, (see, Fig. 5B). The results, shown in Figure 5, demonstrated that the majority of the plasmid DNA, (>90%), about 10 ug, was released from the needles in 1 to 2 min.

Fig 5:

(A) Plasmid released from coated sewing needles after immediate insertion into an agarose gel for increasing time periods.

(B) Plasmid retained by and eluted from coated sewing needles after insertion into an agarose gel for increasing time periods, **(A).**

with each lane representing:

1) 1ug of 1kb DNA ladder, (Promega). 2) Gel release for 15 seconds, (HT).
3) Gel release for 60 seconds, (HT). 4) Gel release for 2 minutes, (HT).
5) Gel release for 5 minutes, (HT). 6) Gel release for 15 minutes, (HT).
7) Empty, 8) Gel release for 15 seconds, (HS). 9) Gel release for 60 seconds, (HS).
10) Gel release for 2 minutes, (HS). 11) Gel release for 5 minutes, (HS).
12) Gel release for 15 minutes, (HS).13) Empty. 14) pGL3CMV plasmid standard – 0.5ug. 15) pGL3CMV plasmid standard – 1.0ug, 16) pGL3CMV plasmid standard – 5.0ug, 17) pGL3CMV plasmid standard – 12.0ug

Example 5, *Improved dose of plasmid coated onto a single hypodermic needle compared to a single sewing needle.*

To increase the dose of plasmid DNA that could be coated and then lyophilized onto a single needle, it was hypothesized that a hollow hypodermic needle would present a greater surface area and accommodate more DNA than a solid sewing needle. 30G hypodermic needles were coated by the same plasmid DNA / needle coating procedure, (using the HS formulation, see Fig. 4b), alongside identical coating procedures for sewing needles and the amount of plasmid eluted was analysed by agarose gel electrophoresis,. Five identical needles of each type were analysed. The results are shown in figure 6 for lanes1) 1ug of 1kb DNA ladder, (Promega), 2), 3), 4),

5) & 6) Eluted pGL3CMV plasmid from coated, methanol washed sewing needles, 7), 8), 9), 10 & 11) Eluted pGL3CMV plasmid from coated, 30G hypodermic needles. 12) pGL3CMV plasmid standard – 5.0ug.

The use of 30G hypodermic needles resulted in at least a two-fold increase in amount of plasmid coated over sewing needles. This suggests that substantially more DNA can be coated onto hollow needles compared to solid needles.

Example 6, *Optimal formulations for plasmid DNA release, after coating and lyophilization, from sewing and hypodermic needles.*

A series of different DNA formulations, lacking a number of individual components of the full HS formulation, (described in Example 3), were compared together with the full HS and LS formulations, for their ability to release DNA after coating and lyophilisation onto needles. A range of needles: size 10 sewing and hypodermics ranging through 21G, 25G, 26G to 30G were compared in this analysis. DNA release was assayed as described in Example 4, Fig. 5 by an 'agarose gel release assay', release time was approximately 2 minutes, followed by gel electrophoresis and such data is shown in Figure 7.

The lanes were pierced with needles coated with the following formulations:

FIG 7A Formulation and needle combinations Lane:-	**FIG 7B**
1) A1	F1
2) A2	Empty
3) A3	F2
4) A4	Empty
5) A5	F3
6) Empty	Empty
7) B1	F4
8) B2	Empty
9) B3	F5
10) B4	Empty
11) B5	G1
12) Empty	Empty
13) C1	G2
14) C2	Empty
15) C3	G3
16) C4	Empty

17) C5	G4
18) Empty	Empty
19) D1	G5
20) D2	Empty
21) D3	H1
22) D4	Empty
23) D5	H2
24) Empty	Empty
25) E1	H3
26) E2	Empty
27) E3	H4
28) E4	Empty
29) E5	H5
30) Empty	Empty
31) Empty	I1
32) Empty	Empty
33) Empty	I2
34) Empty	Empty
35) Empty	I3
36) Empty	Empty
37) Empty	I4
38) Empty	Empty
39) Empty	I5
40 – 45) Empty	Empty
46) pVac1ova plasmid standard – 0.5ug.	pVac1ova plasmid standard – 0.5ug
47) Empty	Empty
48) pVac1ova plasmid standard – 5.0ug	pVac1ova plasmid standard – 5.0ug
49) Empty	Empty
50) 1ug of 1kb DNA ladder, (Promega).	1ug of 1kb DNA ladder, (Promega).

With the formulations described using the following code:

A) 40% sucrose, 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, 2.9% ethanol, (HS),

B) 17.5% sucrose, 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, 2.9% ethanol, (LS),

C) 40% sucrose, 100mM Tris /HCl pH 8.0, 1mM EDTA, 2.9% ethanol, (HS – methionine),

D) 40% sucrose, 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, (HS – ethanol),

E) 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, 2.9% ethanol, (formulation 2),

F) 100mM Tris /HCl pH 8.0, 1mM EDTA, 2.9% ethanol, (formulation 2 - methionine),

G) 100mM Tris /HCl pH 8.0, 1mM EDTA, 10mM methionine, (formulation 2 - ethanol),

H) 40% sucrose in water,

I) 10mM Tris /HCl pH 8.0, 1mM EDTA,

1 - Sewing needles, size 8,

2 - 30G hypodermic needles,

3 - 26G hypodermic needles,

4 - 25G hypodermic needles,

5 - 21G hypodermic needles.

The data suggests that the preferred formulations for optimal DNA release in this assay are those containing sucrose, (either 17.5 %, or 40%) or a full formulation of chelators and free-radical scavengers, (formulation 2) or more preferably a combination of both. The data also demonstrates that DNA release in this assay, for the majority of formulations tested, was best using the sewing needles and hypodermic needles of bore size greater than 26G, (26G optimal), with 30G hypodermic needles being poorest for DNA release in this assay.

Example 7, *Optimal formulations to stabilise plasmid DNA after coating, lyophilization and storage on needles.*

The series of different DNA formulations, lacking a number of individual components of the full HS formulation, (Example 3), as described in Example 6 and Fig. 7, were compared for their ability to stabilise supercoiled plasmid DNA, after coating and lyophilisation onto needles, upon short term storage. Sewing needles, methanol washed, were coated, lyophilised and stored at 4^0C for 7 days. Plasmid DNA was then eluted and recovered in the standard manner and subject to agarose gel electrophoresis, (100V, 100mA for 2 hours), in the absence of intercalating agents, (Sambrook, J. *et al., supra*). The integrity of the eluted plasmid DNA was then monitored after staining with SYBR gold and visualisation under UV light. Such data is shown in Fig. 8, which shows plasmid DNA eluted from methanol-washed sewing

needles after storage which were formulated in a variety of different formulations, A – I, as described in Example 6.

Lanes:-

1) 1ug of 1kb DNA ladder, (Promega).
2) pVac1ova plasmid standard – 5.0ug
3) & (4) Formulation A, (sample lost- lane 3)
5) & (6) Formulation B
7) & (8) Formulation C
9) & (10) Formulation D
11) & (12) Formulation E
13) & (14) Formulation F
15) & (16) Formulation G
17) & (18) Formulation H
19) & (20) Formulation I

The data confirms that formulations containing a combination of sugar with chelating agents and free radical scavengers generate the greatest and most reproducible yield of plasmid DNA when eluted from needles. The data suggests that plasmid DNA is predominately maintained for release in the monomeric supercoiled form only in formulations that contain a combination of sugar with chelating agents and free radical scavengers.

Example 8, *Demonstration of in vivo delivery of functionally active plasmid DNA from coated, lyophilised needles.*

In vivo delivery and luciferase assays.

Plasmid DNA delivery from coated needles was performed into Balb/c x C3H F1 female mice both intramuscularly, (IM), and intradermally, (ID). For IM delivery a DNA coated 30G hypodermic needle was inserted into a pre-shaved femur muscle for 10 to 20 seconds before removal. For ID delivery mice were anaesthetised with isofluorane and a coated 30G hypodermic needle was inserted into a pre-shaved area of abdomen under low powered microscopy for 2 minutes. As positive controls, 10ug of plasmid DNA in saline was injected by standard procedure both IM and ID. Groups

of 10 animals or tissues were analysed versus six positive controls. Mice were sacrificed and samples were removed 48 hours post plasmid delivery and snap frozen in liquid nitrogen.

Muscle or skin samples were thawed to room temperature and disrupted in 500ul of passive lysis buffer, (Promega Corporation, Madison, Wisconsin, USA), using an IKA Labortechnic Ultra turrax T8 polytron. Luciferase enzyme activity was determined using a luciferase assay kit, (Promega). 40µl of the lysate (in duplicate) was assayed together with 200µl of luciferase assay reagent (Promega) in a black 96 well plate, (Nunc). Luciferase activity (RLU) was measured as counts per second in the TopCountNXT HTS scintillation and luminescence counter, (Packard). Total protein concentration was calculated by Coomassie Plus protein assay reagent kit (Pierce) using the manufacturer's protocol. Briefly, 5µl of cell lysate was assayed together with 145µl of water (Sigma) and 150µl of coomasie blue reagent in 96 well flat-bottomed plates (Costar). The absorbance was measured at 595nm on a Molecular Devices Spectra Max 340. Luciferase activity was expressed as relative light units (RLU)/mg of total protein.

Data from such an experiment is shown in Figure 9, where luciferase activity derived from plasmid 'released from coated needles' is compared to that derived from plasmid delivered by standard IM, (Fig. 9A), and ID, (Fig. 9B), injections. Data suggested that at least 1/10 mice were positive for luciferase actvity after ID DNA release from coated needle administration and at least 3/10 mice were positive after the similar IM procedure. This demonstrates the principle that plasmid DNA can be released from these formulations when coated onto needles in a transcriptionally active form to allow expression of an encoded gene or antigen.

Claims

1. A DNA pharmaceutical agent delivery device having at least one skin-piercing element comprising an amorphous solid reservoir medium containing the DNA pharmaceutical agent, and a stabilising agent that inhibits the degradative effects of free radicals.

2. A DNA pharmaceutical agent delivery device as claimed in claim 1 wherein the stabilising agent is one or both of a metal ion chelator and a free radical scavenger.

3. A DNA pharmaceutical agent delivery device as claimed in claim 1, wherein the solid biodegradable reservoir medium containing the pharmaceutical agent is coated externally onto the at least one skin-piercing member.

4. A pharmaceutical agent delivery device as claimed in claims 1, 2 or 3 wherein the solid biodegradable reservoir medium is a polyol.

5. A pharmaceutical agent delivery device as claimed in claim 4, wherein the polyol is a stabilising polyol.

6. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 5 wherein the solid biodegradable reservoir medium is a sugar.

7. A pharmaceutical agent delivery device as claimed in claim 6 wherein the sugar is selected from lactose, sucrose, raffinose or trehalose.

8. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 6 wherein the solid biodegradable reservoir medium forms a glass.

9. A pharmaceutical agent delivery device as claimed in any on of claims 1 to 8 wherein the solid biodegradable reservoir medium releases the pharmaceutical agent within 5 minutes after insertion of the skin-piercing member and solid biodegradable reservoir medium into the skin.

10. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 9 wherein the skin piercing members are dimensioned to deliver the agent into the dermis.

11. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 9 wherein the skin piercing members are dimensioned to deliver the agent into the epidermis.

12. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 11 wherein the skin piercing members microneedles or microblades.

13. A pharmaceutical agent delivery device as claimed in any one of claims 1 to 12, wherein the pharmaceutical agent is a vaccine.

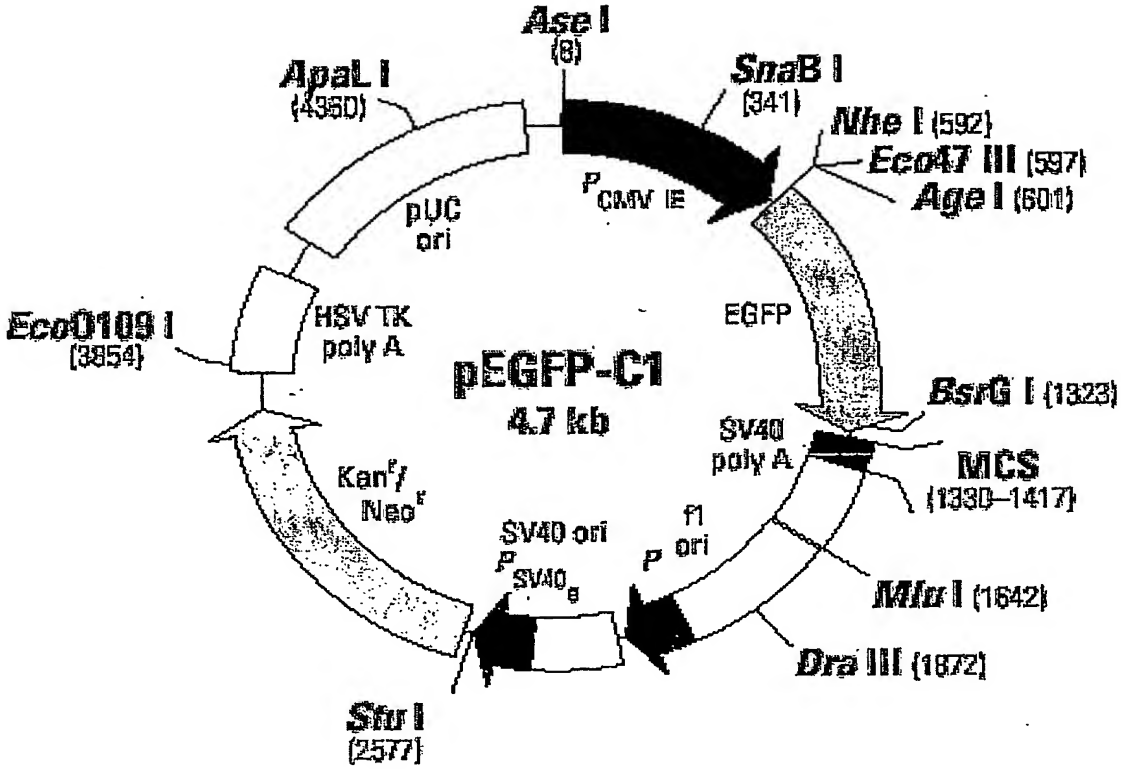
14. A process for the preparation of a DNA pharmaceutical agent delivery device as claimed in claim 1, comprising making a solution of DNA pharmaceutical agent, reservoir medium, and stabilising agent, followed by dipping at least one skin-piercing member into said solution, and allowing the solution to dry onto the skin-piercing member to form a solid biodegradable reservoir medium containing a the pharmaceutical agent.

15. A DNA pharmaceutical agent delivery device as claimed in claim 1 wherein the DNA is stabilised such that it is released in a supercoiled form.

Abstract

The present invention relates to efficient devices for administration of DNA based pharmaceutical agents into the skin of the human body. In particular the present invention provides devices for administration of DNA vaccines into the skin. The present invention provides a DNA agent delivery device having at least one skin-piercing element which comprises a support member coated with an amorphous solid reservoir medium containing the DNA pharmaceutical agent, and a stabilising agent that inhibits the degradative effects of free radicals. Preferably the solid pharmaceutical reservoir medium is an amorphous polyol, and preferably a carbohydrate such as trehalose or sucrose. Preferably the stabilising agent is a free radical scavenger or a metal ion chelator.

FIG 1A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SnaB I
(341)
Nhe I (592)
Eco47 III (597)
Age I (601)
ApaL I
(4360)
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ori
P CMV IE
EGFP
EcoO109 I
(3854)
HSV TK
poly A
pEGFP-C1
4.7 kb
BsrG I (1323)
SV40
poly A
MCS
(1330–1417)
Kan^r/
Neo^r
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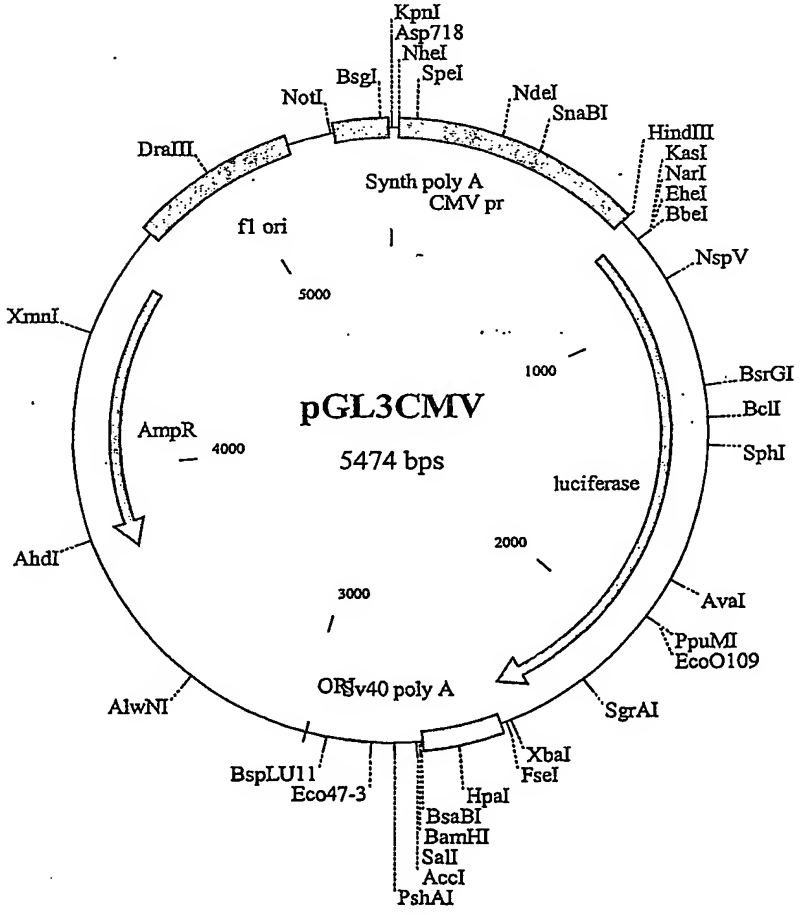
P SV40
f1
ori
P
Mlu I (1642)
Dra III (1872)
Sfu I
(2577)

FIG 1B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BsgI
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DraIII
Synth poly A
CMV pr
f1 ori
NspV
5000
XmnI
1000
BsrGI
BclI
SphI
pGL3CMV
AmpR
4000
5474 bps
luciferase
2000
AhdI
3000
AvaI
PpuMI
EcoO109
ORI
SV40 poly A
AlwNI
SgrAI
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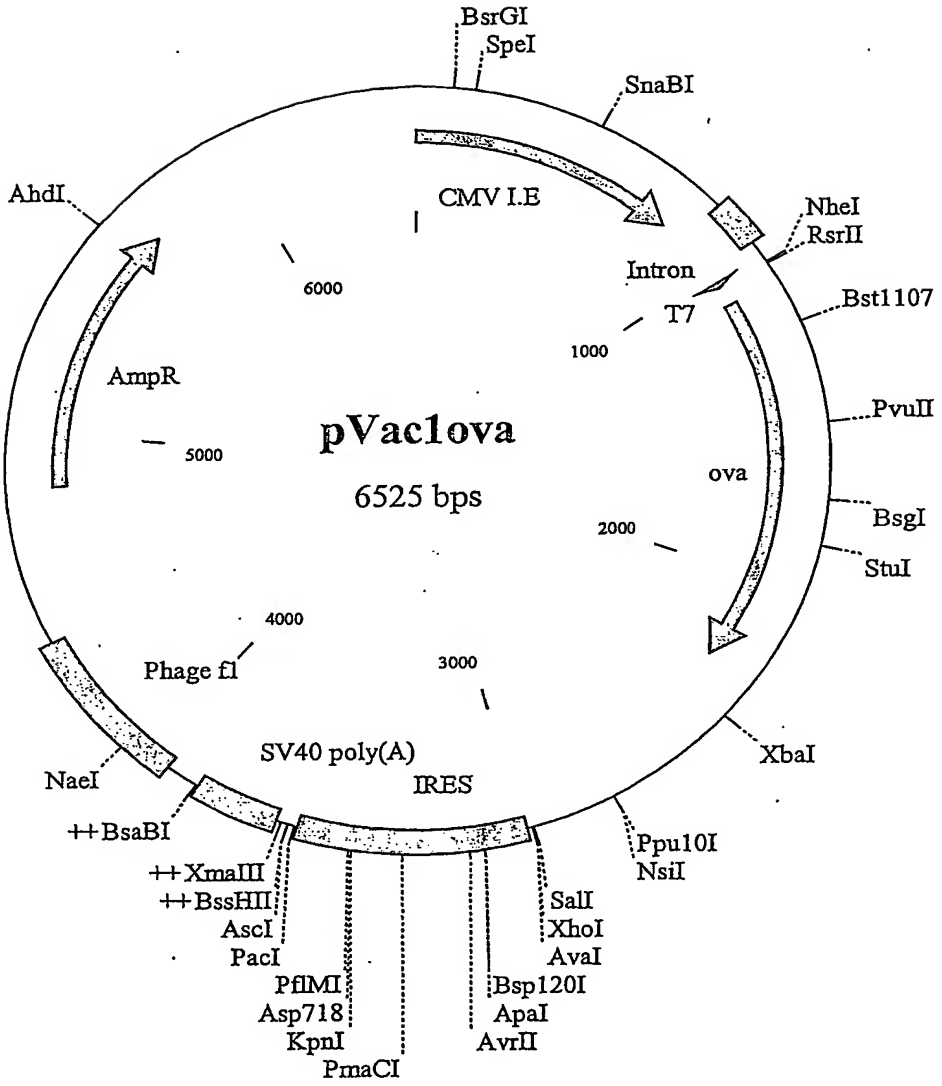
BsaBI
BamHI
SalI
AccI
PshAI

Fig 1C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SpeI
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NheI
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Bst1107
PvuII
BsgI
StuI
XbaI
Ppu10I
NsiI
SalI
XhoI
AvaI
Bsp120I
ApaI
AvrII
PmaCI
KpnI
Asp718
PflMI
PacI
AscI
++BssHII
++XmaIII
++BsaBI
NaeI
SV40 poly(A)
IRES
Phage f1
AmpR
AhdI
CMV I.E
Intron
T7
ova
1000
2000
3000
4000
5000
6000

FIG 2,

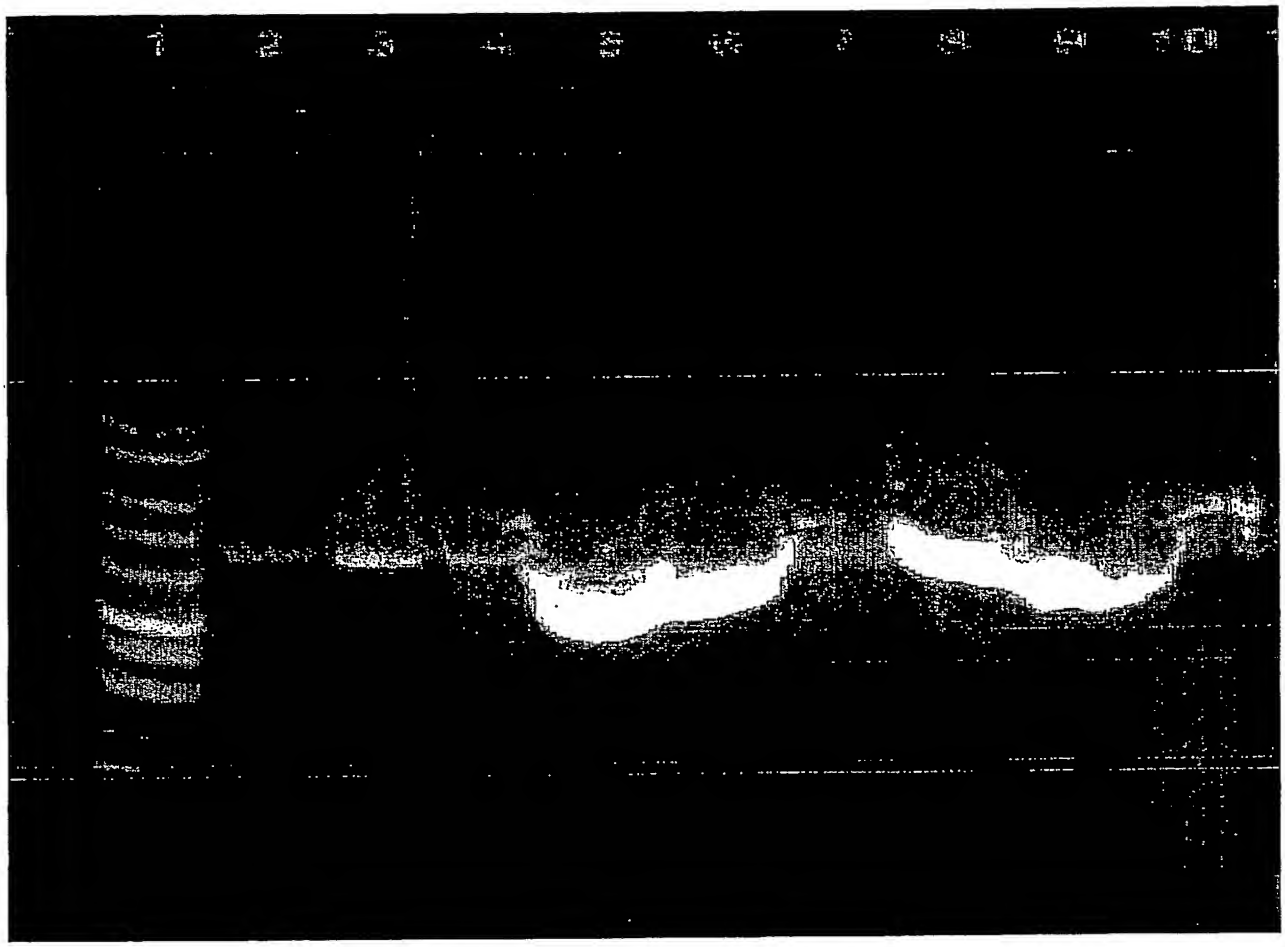


FIG 3A

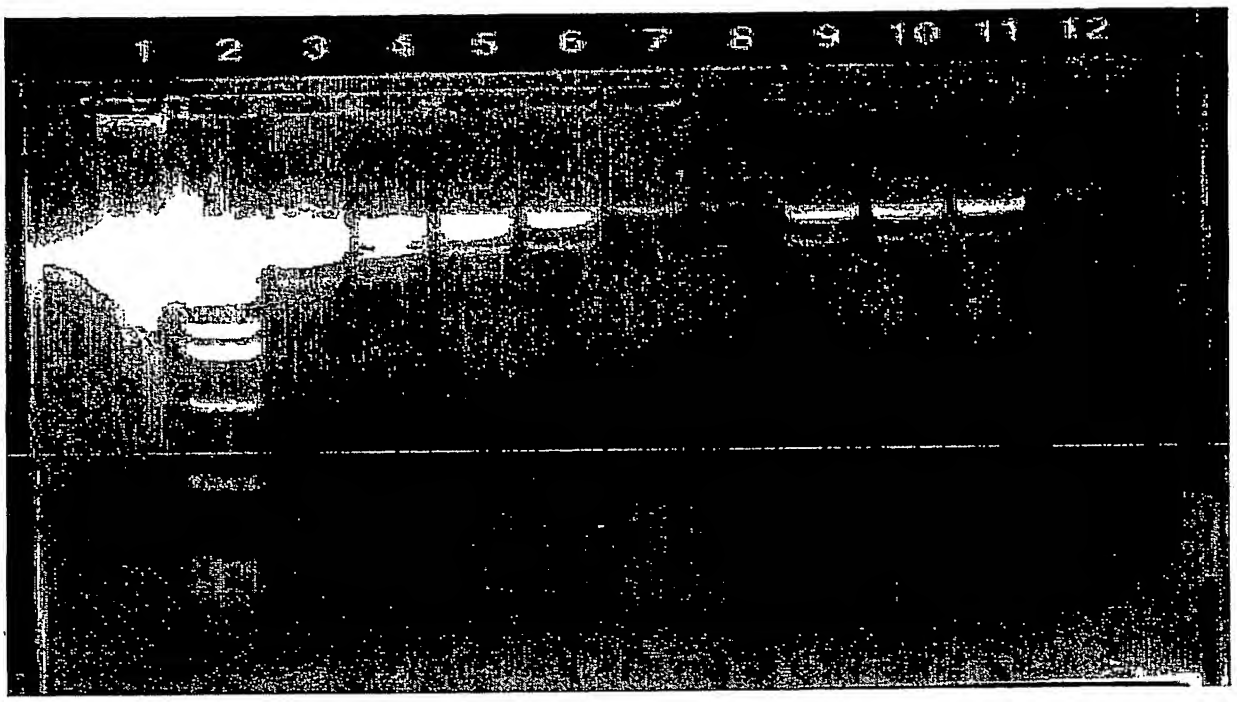


FIG 3B

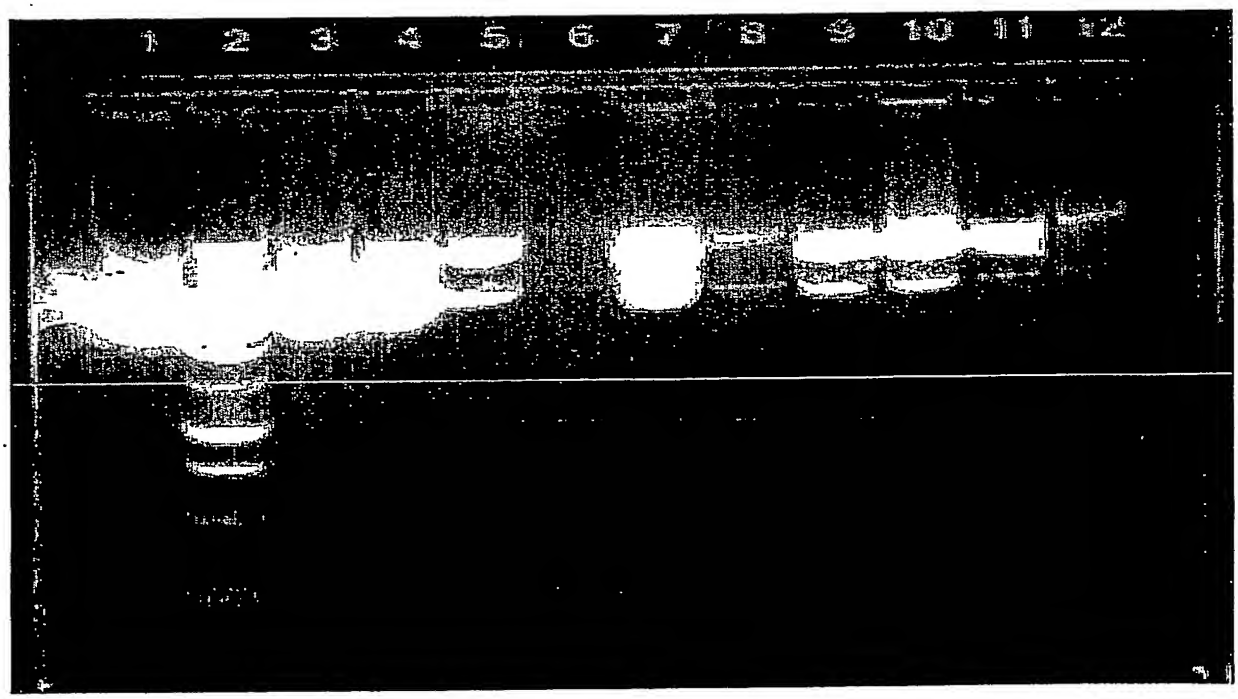


FIG 4A,

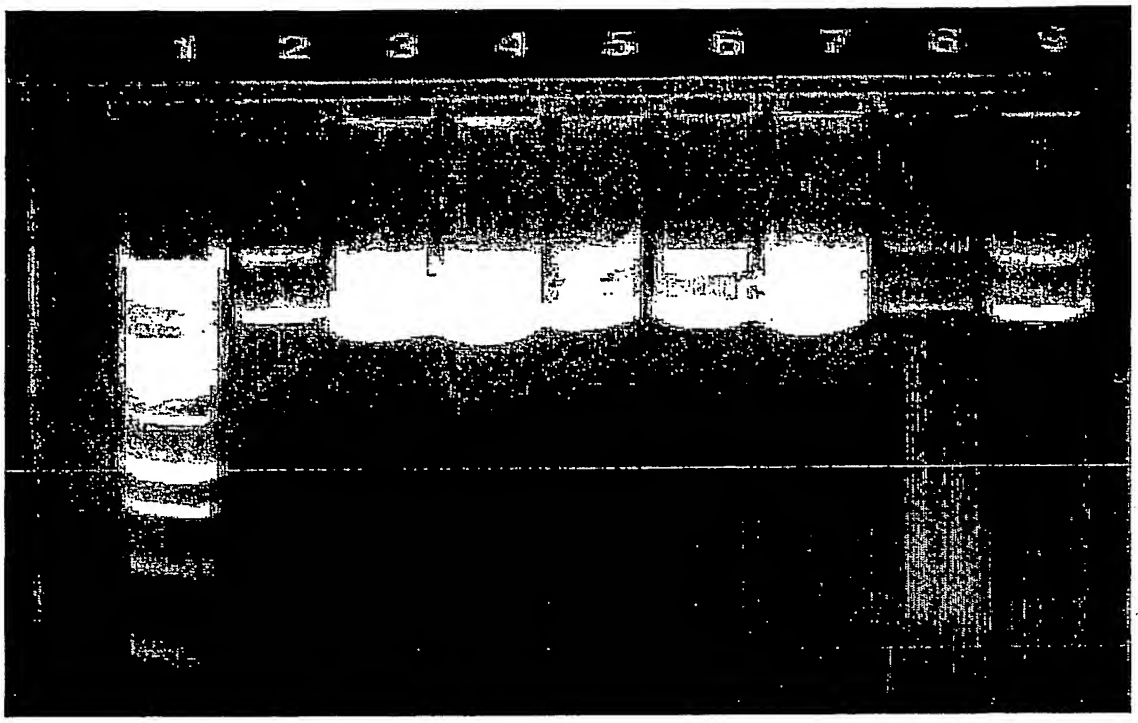


FIG 4B,

FIG 5A,

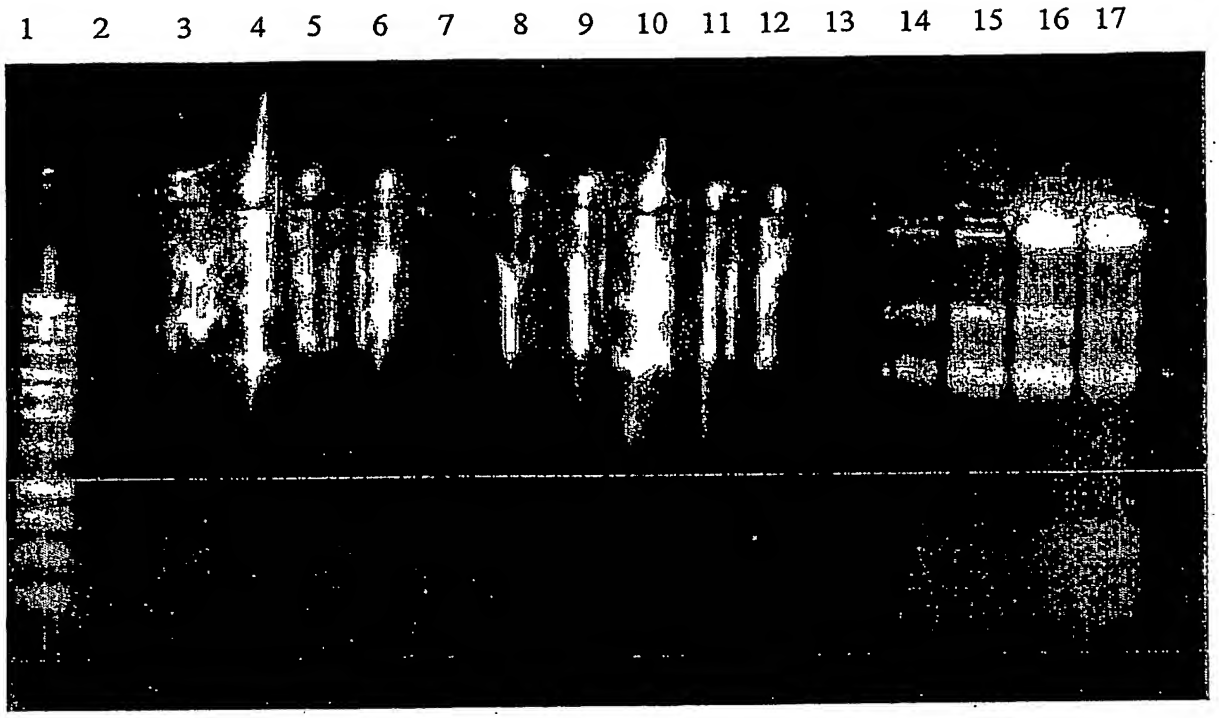


FIG 5B,

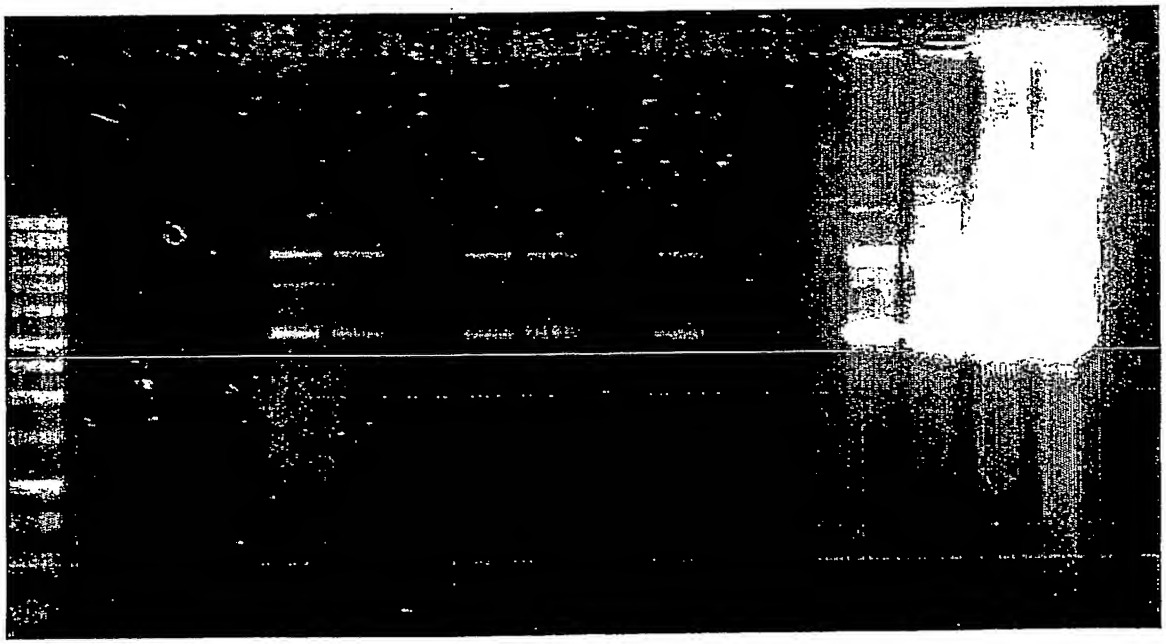


Fig 6,

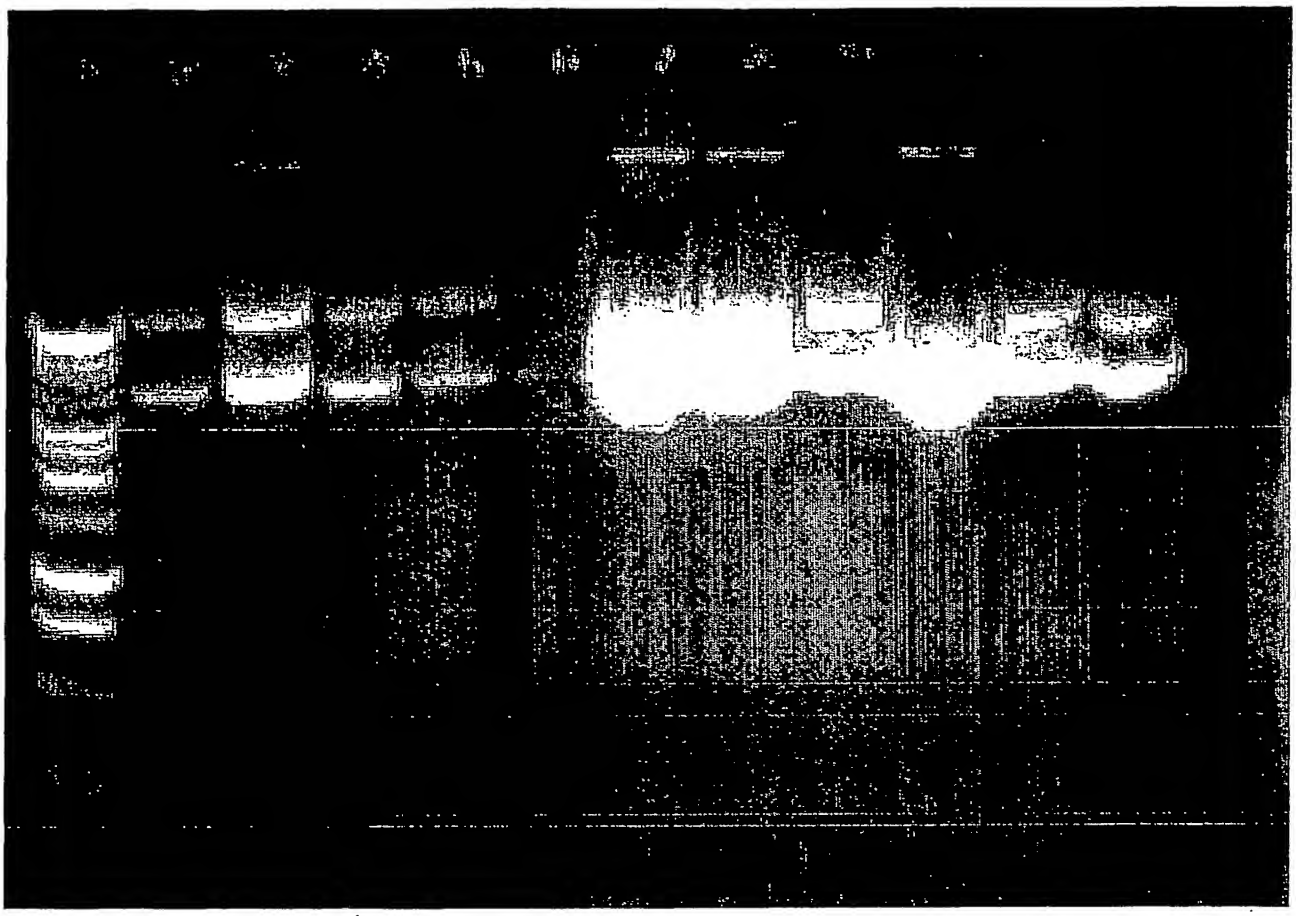


FIG 8,

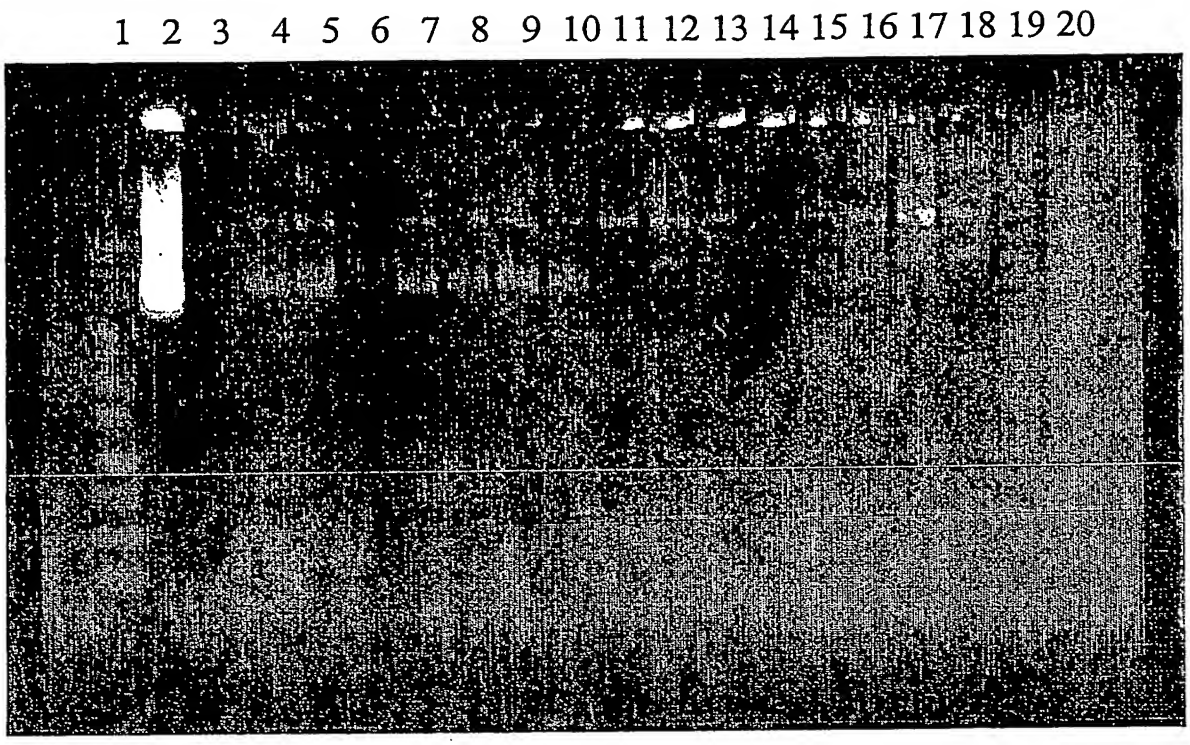
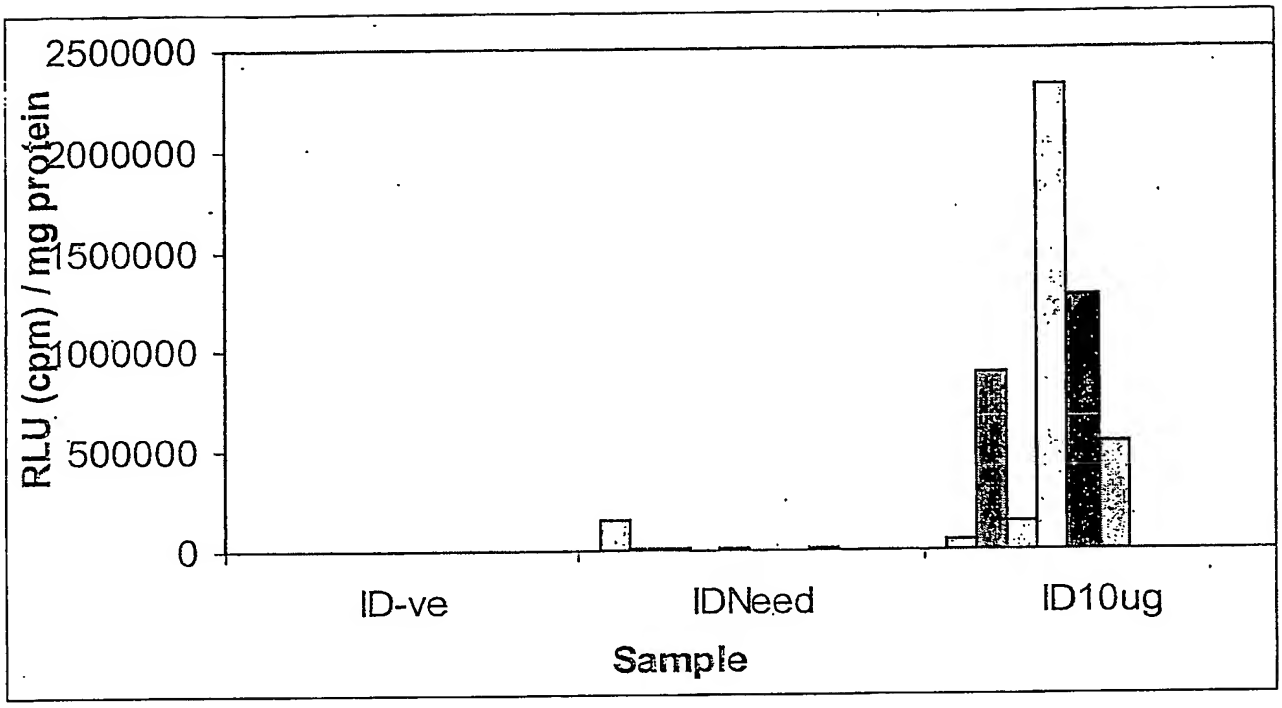


FIG 9

A.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2000000
1500000
1000000
500000
0
RLU (cpm) / mg protein
ID-ve
IDNeed
ID10ug
Sample

B.

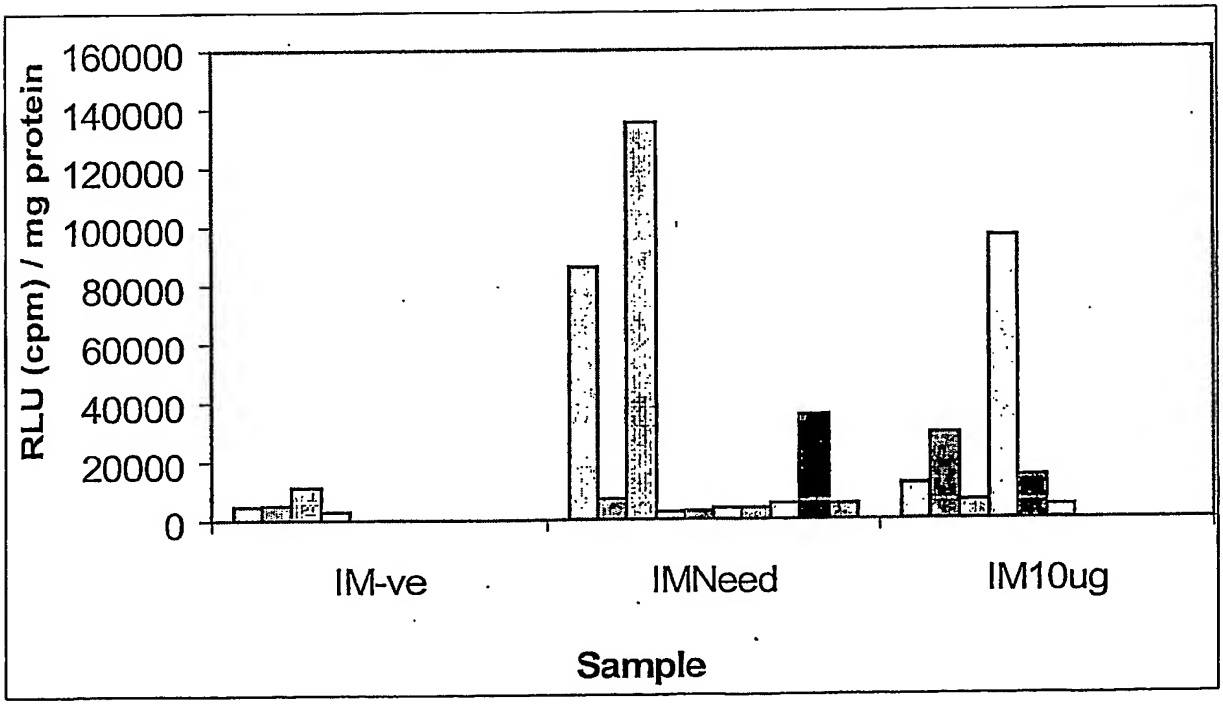
160000
140000
120000
100000
80000
60000
40000
20000
0
RLU (cpm) / mg protein
IM-ve
IMNeed
IM10ug
Sample

FIG 7